REMARKS

I. Preliminary Remarks and Amendments

Claims 1-10 are currently pending. Claims 1-7 and 9-10 are under examination and remain variously rejected under 35 U.S.C.§112, first paragraph, for lack of enablement; §112, second paragraph; and §102(b) over either Orlic et al. (*P.N.A.S.* 98:10344-10349, 2001) or Anversa (Pre-grant Patent Publication No. US 2002/0061587 A1 [05/2002]). Claims 1, 9, and 10 are amended herein. Support for the amendments to claims 1, 9, and 10 is found throughout the specification, including at page 3, line 5, page 5, line 18, page 9, line 8, page 10, line 11, page 19, line 10, and page 20, line 15. Support for new claims 11-16 is found throughout the specification, including at page 6, lines 13-19; page 9, lines 4-11; page 19, lines 9-13; and page 20, lines 15 and 31-32. Accordingly, the amendments do not include new matter. The Applicants do not intend with these or any other amendments to abandon the subject matter of claims previously presented, and reserve the right to pursue such subject matter in duly filed continuing patent applications.

II. Patentability Arguments

A. The Rejections Under 35 U.S.C. §102(b) May Properly Be Withdrawn.

1. Orlic does not anticipate the subject matter of any pending claim.

The Examiner maintained the rejection of claims 1, 2, and 9 under 35 U.S.C. §102(b) for anticipation by Orlic et al., *Proc. Natl. Acad. Sci.* 98:10344-10349, 2001 (hereinafter "Orlic"), because Orlic assertedly discloses the administration of a composition comprising G-CSF three days following coronary artery ligation, demonstrating that this composition was given following ischemia and not merely prophylactically. *See* Office Action at pages 2-4. In response, the Applicants submit that Orlic does not disclose each limitation of any one of the rejected claims, as amended.

Orlic showed that the mobilization of primitive bone marrow cells prior to acute myocardial infarction (AMI), by the prophylactic administration of SCF in combination with G-CSF, daily for five days prior to the ischemic event, and then daily for another 3 more days after the ischemic event, resulted in tissue regeneration in the ischemic site. Thus, Orlic's treatment with SCF in combination with G-CSF was administered **both prior to and after** the ischemic event. Unlike Orlic, the present invention offers an effective treatment for

AMI or arterial occlusion **after** the ischemic event has already occurred. According to MPEP §2111.03, "the transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. *In re Gray*, 53 F.2d 520, 11 USPQ 255 (CCPA 1931). Accordingly, the present invention does not read on Orlic's prophylactic treatment with SCF in combination with G-CSF. Furthermore, Orlic did not demonstrate nor contemplate that treatment with G-CSF alone after an arterial occlusion could have any beneficial effect. In summary, Orlic administered SCF and G-CSF **both prior to** inducing AMI **and** then again **after** the AMI, and observed the effect that this protocol had on tissue regeneration in the heart. Consequently, because Orlic did not treat with G-CSF **only after** AMI or **only after** near or total occlusion of an artery, Orlic does not anticipate claims 1 or 9, as amended.

As a matter of law, a dependent claim incorporates each limitation of a claim from which it depends. 35 U.S.C. §112, fourth paragraph. Claim 2 depends from claim 1 and, as established above, Orlic does not disclose each element of claim 1, as amended. Accordingly, Orlic cannot disclose, expressly or inherently, each limitation of dependent claim 2 and, for that reason, Orlic does not anticipate the subject matter of any dependent claim.

For the foregoing reasons, Orlic does not anticipate the subject matter of any of claims 1, 2, and 9 under 35 U.S.C. §102(b) and, therefore, the rejection should be withdrawn.

2. Anversa does not anticipate the subject matter of any pending claim.

The Examiner maintained the rejection of claims 1-7 and 9-10 under 35 U.S.C. §102(b) for anticipation by Anversa, (Pre-grant Patent Publication No. US 2002/0061587 A1 [05/2002]; hereinafter "Anversa"), because Anversa assertedly discloses giving a composition comprising G-CSF daily for 3 days following coronary artery occlusion. In addition, the Examiner maintained the position that Anversa discloses a method of reperfusion therapy comprising administering G-CSF and other cytokines which assertedly induce stem cell mobilization, in a treatment directed to ischemia and reperfusion. The Examiner asserted that Anversa teaches administering compositions comprising G-CSF, as well as G-CSF alone, and administering said compositions following ischemia, which anticipates the instant claims. *See* Office Action at pages 4-6. In response, the Applicants

submit that Anversa does not disclose each limitation of any one of the rejected claims, as amended.

Like Orlic, Anversa showed that the mobilization of stem cells prior to acute myocardial infarction (AMI), by the prophylactic administration of SCF in combination with G-CSF, daily for five days prior to the ischemic event, and then daily for another 3 more days after the ischemic event, resulted in increased survival in mice and myocardial regeneration. Thus, Anversa's treatment with SCF in combination with G-CSF (like that of Orlic as set out above) was administered **both prior to** the ischemic event **and after** the ischemic event. Unlike Anversa (and Orlic), the present invention offers an effective treatment for AMI or arterial occlusion **after** the ischemic event has already occurred. As set out above, "the transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. *In re Gray*, 53 F.2d 520, 11 USPQ 255 (CCPA 1931), MPEP §2111.03. Accordingly, the present invention does not read on Anversa's prophylactic treatment with SCF in combination with G-CSF. Furthermore, Anversa did not demonstrate nor contemplate that treatment with G-CSF alone after an arterial occlusion could have any beneficial effect. In summary, Anversa (like Orlic) administered SCF and G-CSF **both prior** to inducing AMI **and** then again **after** the AMI, and observed the effect that this protocol had on survival and myocardial regeneration. Consequently, because Anversa did not treat with G-CSF **only after** AMI or **only after** near or total occlusion of an artery, Orlic does not anticipate claims 1-7 and 9-10, as amended.

Although Anversa hypothesizes that the stem cells "home to the damaged area of the heart" and migrate "into the infracted area," as pointed out by the Examiner (*see* Office Action at page 6), Anversa contemplates the proliferation and mobilization of stem cells into the blood stream prior to inducing AMI. Thus, Anversa's method involves treatment with SCF and G-CSF **both prior to and after** inducing AMI for the mobilization of stem cells. Anversa does not disclose the use of G-CSF alone in a method of reperfusion therapy for the treatment of AMI as is claimed in the present invention. Anversa's method comprises the delivery of somatic stem cells, alone or in combination, with cytokines, including SCF, G-CSF, GM-CSF, IL-3, etc. (*see* Anversa at p. 1, paragraph [005]). Anversa teachs in paragraph [005] that the methods and/or pharmaceutical compositions of his invention comprise an effective amount of stem cells, alone or in combination with, a cytokine. In fact, none of Anversa's methods involve the treatment of mammals with G-CSF in conjunction with reperfusion therapy after myocardial infarction to reduce heart damage (*see* Anversa's

Examples 1-7; paragraphs [0160-0201]. Anversa's Example 2, for example, uses only the prophylactic treatment of SCF in combination with G-CSF for five days prior to an induced AMI to improve survival, promote myocardial regeneration, reduce infarct size, and increase posterior wall thickness (same results as published by Orlic as discussed above). Anversa did not disclose the use of G-CSF in a reperfusion therapy method for improved patient outcome or increased ventricular wall thickness.

Although Anversa's methods and compositions may assertedly induce stem cell mobilization and migration and aid in the regenerative process of the heart following ischemia and reperfusion, Anversa does not anticipate the present invention. Anversa showed that the mobilization of primitive bone marrow cells **prior to** AMI, by the prophylactic administration of SCF in combination with G-CSF (prior to the ischemic event), resulted in a significant degree of tissue regeneration in the ischemic site. Anversa also does not disclose the use of G-CSF alone in the treatment of an occlusion in an artery. Anversa did not demonstrate nor contemplate that treatment with G-CSF alone **after** an arterial occlusion could have any beneficial effect. Thus, Anversa does not anticipate claims 1, 9, or 10.

As set out above, a dependent claim incorporates each limitation of a claim from which it depends. Thus, claims 2-7 depend from claim 1 and, as established above, Anversa does not disclose each element of claims 1, 9, or 10, as amended. Accordingly, Anversa cannot disclose, expressly or inherently, each limitation of any of dependent claims 2-7 and, for that reason, Anversa does not anticipate the subject matter of any dependent claim.

For the foregoing reasons, Anversa does not anticipate the subject matter of any of claims 1-7 and 9-10 under 35 U.S.C. §102(b) and, therefore, the rejection should be withdrawn.

B. The Rejections Under 35 U.S.C. §112, First Paragraph, May Properly Be Withdrawn.

The Examiner maintained the rejection of claims 1 and 5 under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for a method of improving wall thickness following ischemia and reperfusion, does not assertedly provide

enablement for the broad claim of a method of reducing all forms of heart damage following ischemia and reperfusion. In response, the Applicants respectfully traverse the rejection.

The Applicants reassert their previous position that the specification enables a reduction in heart damage, as originally claimed. However, in order to expedite prosecution, the Applicants have amended claim 1 to recite "to reduce infarct-related myocardial tissue damage." Thus, the Applicants submit that the rejection of claim 1 for lack of enablement has been overcome by amendment and should properly be withdrawn.

Finally, the Examiner maintained the rejection of claim 5 for asserted lack of enablement because the method of claim 5 uses a composition comprising G-CSF and "numerous cytokines that are proinflammatory, such as IL-8." The Examiner asserted that IL-8 is an inflammatory cytokine that is upregulated during ischemia and reperfusion, including coronary artery bypass (Vallely et al., *J. Thorac. Cardiovasc. Surg.* 124:758-767, 2002, hereinafter "Vallely"), and, therefore, this cytokine would actually increase heart damage by increasing inflammation (*see* previous Office action, mailed 10/20/04, at page 4). The Examiner indicated that until discrepancies have been worked pertaining to certain cytokines having proinflammatory properties and the ability to promote growth and proliferation of hematopoietic cells it would be considered an invention to experiment, not a patentable invention (*see* Office action at page 8) The Applicants respectfully traverse this rejection.

Although the Examiner has suggested that Vallely indicates that IL-8 is pro-inflammatory and suggests that this cytokine would increase heart damage, the Applicants submit that there have been other reports, e.g., Laterveer et al. (*Exp. Hematol.* 24:1387-1393, 1996; submitted previously as Appendix A in response mailed 1/19/2005), which have shown that IL-8 induces instant mobilization of hematopoietic progenitor cells in mice and primates. The Applicants also submit that the upregulation in IL-8 reported by Vallely could have been the result of an expansion of the total bone marrow cell number after surgery and a subsequent release of stem cells into the blood circulation (Grzelak et al., *Eur. Surg. Res.* 30:198-204, 1998; hereinafter "Grzelak", enclosed as **Appendix A**). Grzelak, *supra*, also reported that IL-8 possesses progenitor cell mobilization properties. Thus, the fact that IL-8 mobilizes progenitor cells, a fact that is well-established in the art, suggests that IL-8 may also be useful in the mobilization of progenitor cells in the repair or prevention of heart damage after an AMI. Thus, one of skill in the art would have known, at the time the

application was filed, that IL-8 promotes progenitor cell mobilization. Moreover, the Patent Office has issued numerous patents containing claims, which support a role for IL-8 in the growth and proliferation of hematopoietic cells (see U.S. Patent Nos. 5,437,994; 5,635,386; 5,646,043; 6,012,450; 6,207,802; 6,248,319; 6,326,198; 6,436,387; and 6,852,313). Therefore, there is ample evidence in the art that one would use IL-8 in a composition to promote growth and proliferation of hematopoietic cells. The Applicants reassert their position that one of skill in the art might use IL-8 in conjunction with G-CSF in the methods of the invention. There can be no doubt that one of skill in the art could easily determine the effect of treatment with IL-8 in combination with G-CSF on heart damage using the methods of the invention and using nothing more than routine experimentation.

In view of the amendments and the above discussion, Applicants respectfully request that the rejection of claims 1 and 5 for lack of enablement have been rendered moot-in-part or overcome in-part and should be withdrawn.

III. Conclusion

In view of the amendments and remarks made herein, the Applicants respectfully submit that claims 1-7 and 9-10 are in condition for allowance and respectfully request expedited notification of same. Should the Examiner have any questions, he is welcomed to contact the undersigned at the telephone number below.

Respectfully submitted,

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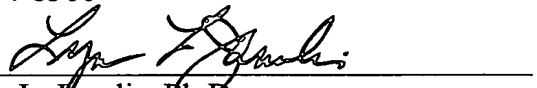
By: 

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May 20, 2005

Original Paper

European Surgical Research

Eur Surg Res 1998;30:198–204

Received: October 30, 1996
Accepted: August 19, 1997

Surgical Trauma Evokes a Rise in the Frequency of Hematopoietic Progenitor Cells and Cytokine Levels in Blood Circulation

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Abstract

Alterations in the mononuclear cell populations in the blood circulation are among the most characteristic changes after surgical trauma. They reflect changes in the hematopoietic compartment which develop following surgery. The process of mobilization and differentiation of the hematopoietic population is regulated by cytokines known as growth factors for stem and progenitor cells (SCF, IL-1, IL-3, IL-6, IL-11, TNF, CSFs). Our question was whether operative trauma resulted in the release of the hematopoietic progenitor cells to the blood circulation and an increase in the blood level of cytokines participating in hematopoiesis. The studies were carried out in patients with chronic cholelithiasis, undergoing elective open cholecystectomy under general anesthesia. An increase in the frequency of circulating CD34+ hematopoietic progenitor cells was seen between days 3 and 7 after surgery. Moreover, a significant increase in the percentage of immature cells of myeloid lineage (CD13+, CD14+, CD33+) was seen on the 1st and 3rd postoperative days. This could be the result of an expansion of the total bone marrow cell number after surgery and a subsequent release of these cells into the blood circulation. The changes in blood cell populations were accompanied by an increase in IL-6 on days 1, 3, and 7 following surgery, in IL-6sR on days 10 and 14 and in IL-8 on days 1 and 3. No significant changes in IL-1α, IL-1β, IL-3 and IL-11 were noted. A small rise in GM-CSF was noted in few patients on the 3rd and 7th postoperative days. It is known that IL6 is involved in hematopoiesis, that the IL-6-IL-6sR complex may induce both proliferation and differentiation of hematopoietic progenitor cells and that IL-8 possesses progenitor cell mobilization properties. The appearance of hematopoietic progenitor cells in the blood following surgery may represent a process for the expansion of the immune cell pool after trauma and maintaining of the reserves at a certain level.

Key Words

Surgical trauma
Progenitor cells
Cytokines

Introduction

Surgical trauma is followed by significant alterations in the immune response resulting in reduced immunoresponsiveness [1]. The decreased postsurgical immunoresponsiveness may be important in clinical practice, due to its contribution to increased susceptibility to local and systemic infections [2, 3], delayed wound healing [4] and accelerated

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local growth and/or dissemination of tumors [5].

One of the most characteristic immune changes after surgical trauma is alteration in the frequency of various mononuclear cell populations in the blood [1, 2], reflecting changes in the hematopoietic compartment. Release of mature form of cells and the accelerated maturation in the bone marrow and lymphoid organs, followed by the release of immature cells in the case of prolonged stress, is commonly seen. Cytokine responses of stem cell factor (SCF), IL-1, IL-3, IL-6, IL-11, TNF and CSFs, participating in hematopoiesis and evoked by trauma, may play an important role in this process.

The presence of hematopoietic progenitor cells (HPC) in peripheral blood has been well documented both in humans and animals [6] and these cells seem to represent an important part of the feedback-regulated hematopoietic cell renewal system [7]. Elevated levels of circulating HPC are seen after chemotherapy, cytokine therapy, endotoxin challenge, *Bordetella pertussis* vaccination, ACTH administration, exercise and administration of steroids, suggesting that this is a general response to hematopoietic stress [7]. In humans, the CD34 cell surface surface marker has been shown to characterize cells with hematopoietic activity. The CD34 antigen is a transmembrane glycoprotein of 110–120 kD [8] which is present on approximately 0.5–3% of bone marrow cells and less than 1% of peripheral blood mononuclear cells. Its expression is highest on the earliest progenitor cells and is progressively lost as these cells mature [9]. The CD34+ cell population comprises virtually all hematopoietic progenitors, forming both unipotent and multipotent cell colonies, including granulomonocytic (CFU-GM), erythroid (BFU-E), megakaryocytic (CFU-Mk), multilineage (CFU-mix) and blast (CFU-blast) cell colonies [10].

Our question was whether surgical trauma evoked changes in the hematopoietic centers resulting in a release of HPC (CD34+ cells) into the circulation. Systemic production of cytokines, which might be responsible for the release of HPC to the blood, was also studied. Furthermore, the blood distribution of other immune mononuclear cells which might be affected by surgical trauma, as myeloid cells, T cells, B cells and T memory cells, was examined.

Material and Methods

Patients

Studies were carried out in 10 patients (5 males and 5 females) with chronic cholelithiasis, aged 25–65, undergoing elective open cholecystectomy under general anesthesia. Cholecystectomy was chosen for the studies as it produces moderate trauma with similar damage of tissues and duration of operative stress in each case. The operation was performed in the 'silent' period during which no clinical signs of inflammation were present. No blood transfusions or antibiotics were given prior, during and after the operation. The postoperative course was uneventful. Balanced anesthesia with nitric suboxide and oxygen (75/25%) was performed. Analgesia was induced using Phentanyl whereas relaxation was performed using Norcuronium.

The protocol of the study was approved by the Ethics Committee of Experimental and Clinical Medical Research Center, Warsaw, Poland, and each patient gave her/his written informed consent.

Blood Samples

Heparinized venous blood samples were taken from the patients on the day preceding the operation and on days 1, 3, 7, 10 and 14 after the operation. Blood samples from 15 healthy volunteers, aged 25–60, were obtained for control purposes.

Plasma Samples for Cytokine Measurements

Plasma samples were prepared by centrifuging the blood at 2,000 rpm for 10 min. They were stored at −20°C until used.

Isolation of PBM Cells

Peripheral blood mononuclear (PBM) cells were isolated from heparinized venous blood samples by

centrifuging the blood on Lymphoprep (Nyegaard, Oslo) at 1600 rpm for 35 min. Cells from the interface were collected and washed three times in RPMI-1640 medium (Gibco, Glasgow, UK).

Flow Cytometry

Enumeration of PBM cell subpopulations was performed by flow cytometry using a panel of monoclonal antibodies obtained from DakoPatts (Denmark). The following antibodies against specific antigens were used: anti-CD34 (TUK3, hematopoietic progenitor cells), of IgG_3 isotype, anti-CD3 (T cells), anti-CD4 (helper/inducer T cells), anti-CD8 (suppressor/cytotoxic T cells), anti-CD13 and anti-CD33 (immature myeloid lineage cells, monocytes), anti-CD22 (B cells), anti-CD23 (B cell subset, Fc-RII), anti-CD38 (stem cells, pre-B cells, plasma cells, monocytes, activated T cells), anti-CD71 (proliferating cells, transferrin receptor), anti-HLA-DR (B cells, Langerhans cells, dendritic cells, myeloblasts, monocytes), all of IgG_1 isotype, anti-CD14 (monocytes, macrophages), anti-TdT (hematopoietic precursor cells, thymocyte cells), anti-CD45 RO (T memory cells), all of IgG_2 isotype. Both FITC- and PE-labelled as well as unlabelled antibodies were used and, respectively, either a direct or an indirect method of staining was employed. The respective isotype controls were also used. PBM cells (5×10^5/100 µl) were incubated with a 1:10 dilution of monoclonal antibody at 4°C for 30 min and then washed twice with PBS containing 2% fetal calf serum (FCS) (Gibco). In case of the direct staining method, cells were suspended in phosphate-buffered (PBS) (containing 1% paraformaldehyde) and stored at 4°C in the dark until analysis. In case of the indirect staining, the 1:20 dilution of fluorescein conjugated $(Fab')_2$ goat anti-mouse IgG was added to each tube and another 30-min incubation on ice was performed. After incubation, samples were washed twice in PBS-FCS, suspended in PBS (with paraformaldehyde) and stored at 4°C in the dark until analysis. The cell subpopulations were analyzed on FACStar flow cytometer (Becton-Dickinson). Lymphocytes were gated by low-angle forward and right-angle light scatter properties. Ten thousand cells were counted from each sample. The percentage of cells reactive with each monoclonal antibody was determined by comparison of fluorescence-labeled cells with cells that had been labeled with isotype controls.

Cytokine Measurements

Plasma concentrations of IL-1α, IL-1β, IL-3, IL-6, IL-6sR, IL-8, GM-CSF and IL-11 were measured by a two-site enzyme immunometric assay (Quantikinine for IL-1α, IL-1β, IL-6, GM-CSF and IL-11; Quantikinine HS, high sensitivity, for IL3, R&D Systems, British Bio-technology Ltd., Abingdon, Oxon., UK). Each plasma sample was examined in duplicate. Intra- and interassay precisions for IL-1α were 1.5 and 4.4%, at IL-1α concentrations of 28.6 and 27.9 pg/ml, respectively, for IL-1β 8.5 and 8.4%, at IL-1β concentrations of 18.9 and 29.8 pg/ml, for IL-3 4.5 and 5.5%, at IL-3 concentrations of 15.0 and 18.0 pg/ml, for IL-6 4.3 and 6.3%, at IL-6 concentrations of 16.8 and 17.2 pg/ml, for IL-6sR 8.6% and 6.4%, at IL-6sR concentrations of 134.0 and 130.0 pg/ml, for IL-8 3.9 and 12.2%, at IL-8 concentrations of 96.9 and 91.0 pg/ml, for GM-CSF 2.2 and 7.3%, at GM-CSF concentrations of 17.8 and 20.6 pg/ml and for IL-11 4.4 and 8.0%, at IL-11 concentrations of 50.0 and 51.0 pg/ml. The lower limit of sensitivity of the assay for plasma/serum samples was 0.2 pg/ml for IL-1α, 0.3 pg/ml for IL-1β, 0.23 pg/ml for IL-3, 0.7 pg/ml for IL-6, 140.0 pg/ml for IL-6sR, 18.1 pg/ml for IL-8, 2.8 pg/ml for GM-CSF and 4.0 pg/ml for IL-11.

Statistical Evaluation

All data were expressed as means ± standard deviation. For evaluation of the statistical significance of differences between preoperative and postoperative results, the Wilcoxon matched pairs signed test with the Bonferroni correction [11] was used (for 5 pairwise comparisons; postoperative days 1, 3, 7, 10 and 14 vs. day −1 and postoperative day 3 vs. day −1).

Results

Mononuclear Blood Cell Phenotype Analysis

No significant differences between preoperative values in patients and the control group in the percentage of any of the examined PBM cells subsets were noted. No circulating CD34+ cells were detected on day 1 after surgery whereas increased numbers were apparent on days 3 and 7 ($p < 0.05$) (table 1). Preoperative values were restored at day 14. In 1 patient, the percentage of CD34+ cells reached 9.1% on day 3 after surgery. A slight increase in the TdT+ cell population was also noted, but the measured values were very low and did not exceed 1% (data not presented). An evident rise in the mean percentage of

Table 1. The percentages of peripheral blood cell populations in patients following elective open cholecystectomy (n = 10)

Specificity	Days				
	−1	+1	+3	+7	+14
CD34	0.7±0.2	0	3.2±1.1**	2.1±0.8*	0.4±0.2
CD13	10.1±2.2	28.3±6.9*	16.6±6.5*	13.6±3.4	10.2±1.8
CD14	6.1±2.0	20.2±5.2*	16.2±3.2*	11.2±4.1*	7.6±1.6
CD33	11.1±3.5	33.8±6.8**	23.1±5.3**	12.2±4.4	9.2±1.6
CD38	32.8±4.7	62.2±7.9*	48.4±7.3**	39.4±5.2	38.8±5.1
CD3	66.7±6.2	59.5±5.4*	54.1±2.0**	60.3±5.9	65.8±4.0
CD4	38.2±6.3	29.5±5.7*	34.4±4.1	41.8±6.8	36.5±5.2
CD8	23.1±4.5	19.4±5.6	18.4±3.1	18.0±4.5	21.4±2.3
CD45RO	37.0±7.9	52.8±8.8**	37.7±7.4	41.2±4.9	37.1±5.7
CD22	10.0±3.8	10.3±2.6	11.4±3.7	11.0±2.9	10.7±2.6
CD23	6.2±2.5	5.0±2.2	6.5±2.0	7.1±2.8	7.1±0.7
HLA-DR	12.5±3.6	28.9±7.9*	20.6±6.5*	24.0±4.9*	15.0±4.1

Values are mean percentages ± SD.
* $p < 0.02$, ** $p < 0.05$, statistically significant vs. preoperative values.

CD38+ cells was observed ($p < 0.02$) (table 1). Elevated percentages of CD13+ cells ($p < 0.02$), CD14+ ($p < 0.02$) and CD33+ ($p < 0.05$) were observed in the PBM cell population (table 1). More HLA-DR+ cells were noted on days 1, 3 and 7 after surgery ($p < 0.02$), both in the PBM and lymphocyte cell populations. Moreover, an increased percentage of CD45RO+ cells was seen ($p < 0.05$) (table 1). There were slight decreases in percentages of CD3+ and CD4+ cells on day 1 after surgery ($p < 0.02$) (table 1). A slight increase in the percentage of CD71+ cells was also noted (data not presented). No changes were seen in the CD22+ and CD23+ cell population (B cells) (table 1).

Cytokine Measurements

Plasma levels of all examined cytokines in patients before the operation did not differ from those noted for normal volunteers. No increases in the plasma levels of IL-1α, IL-1β and IL-3 were seen in patients between days 1 and 14 after surgery (data not presented). There was, however, a significant increase in IL-6 on days 1, 3 and 7 ($p < 0.05$) (table 2). Preoperative values were restored on day 10. The plasma levels of IL-6sR were found increased on days 10 and 14 ($p < 0.05$) (table 2). Furthermore, an elevation in the plasma level of IL-8 was seen on day 1 (table 2). There was an increase in GM-CSF in 3 out of 9 patients on day 3 and in IL-11 in 1/9 patients on days 3 and 7. In other patients, no significant changes in GM-CSF and IL-11 were noted during the entire observation period (data not presented).

Discussion

The obtained results indicate that surgical trauma of an intermediate degree (elective cholecystectomy, general anesthesia, no blood

Table 2. The plasma levels of IL-6, IL-6sR and IL-8 in patients following elective open cholecystectomy (n = 10)

Cytokine	Days					
	-1	+1	+3	+7	+10	+14
IL-6	4.1±3.0	96.7±74.0*	37.1±31.9*	16.9±10.9*	11.6±10.5	8.8±2.2
IL-6sR	33.7±4.7	29.6±3.8	32.5±4.7	36.8±5.6	40.3±5.1*	40.1±5.5*
IL-8	32.3±26.3	65.4±39.9*	58.9±46.9	29.7±19.8	24.2±21.2	32.8±23.2

Values are means ± SD pg/ml for IL-6 and IL-8 and ng/ml for IL-6sR.
* $p < 0.05$, statistically significant vs. preoperative values.

transfusion, uneventful postoperative course) causes a release of hematopoietic progenitor and immature myeloid lineage cells (CD34+, TdT+, CD38+, CD13+, CD14+, CD33+) from the bone marrow to the blood circulation between days 1–7 after surgery. The CD34+ and TdT+ specificities are present on stem and immature cells of all lineages. Their appearance might be a result of an expansion of the total bone marrow cell number following surgery and a subsequent release of these cells into the blood circulation. Expansion of this population might be a homeostatic process to maintain hematopoietic reserves at a certain level. A significant increase in the frequency of cells of myeloid lineage (CD13+, CD14+, CD33+; monocytes, immature myeloid cells) was also seen in the early postoperative period. The mobilization of reserves of phagocytic and antigen-presenting lineage cells might serve to eliminate host cellular antigens released from the damaged tissues and to aid the healing process. The percentage of all T cells (CD3+) and T cell subsets (CD4+ and CD8+) decreased after surgery, but a significant rise in the percentage of CD45RO+ T memory cells was seen in the peripheral blood on day 1. This may reflect a rapid redistribution of the memory population between different lymphoid compartments and its influx to the peripheral blood. It is likely that the memory cells migrate to the site injury having a predilection to extravasate [12].

Cytokine response evoked by trauma might play a critical role in the process of mobilization of bone marrow cells. Several studies have demonstrated elevated cytokine levels (SCF; leukemia inhibitor factor, LIF, IL-3, IL-6, GM-CSF, G-CSF, M-CSF, IL-8, IL-11) in plasma of patients following chemotherapy and transplantation of bone marrow of peripheral blood stem cells and their kinetics was strictly related to hematopoietic recovery [13]. Since surgical trauma is associated with systemic production of various cytokines possessing hematopoietic properties, they may participate in HPC release and/or proliferation. We have found a significant increase in IL-6 plasma levels on days 1, 3 and 7, of IL-6sR at days 10 and 14 after surgery and that of IL-8 on days 1 and 3. There were no changes in IL-1α, IL-1β, IL-3 and IL-11 systemic production, whereas in few patients a slight elevation in GM-CSF production was noted on days 3 and 7.

The clinical implications of the increased levels of circulating IL-6 in patients following surgery are not yet clear. IL-6 is known to be involved in hematopoiesis, especially in its early stages [14]. It was found to enhance the

proliferation of multipotential hematopoietic cells in the human, murine and rat systems [14]. Moreover, IL-6 synergic action with other hematopoietic growth factors as IL-3, IL-4, GM-CSF and M-CSF has been described [15–17]. It has been suggested that IL-6 causes a shift of the hematopoietic stem cells from the G_0 to the G_1 stage of the cell cycle where they become more responsive to the effects of additional hematopoietic growth factors [18]. The question arises whether the increased IL-6 levels in the blood following operative trauma, might evoke a rise in the CD34+ hematopoietic progenitor cell population in the blood circulation. Data obtained from the animal studies on the in vivo administration of IL-6 to mice with myelosuppression demonstrated that IL-6 might enhance multilineage hematologic recovery following either chemotherapy or radiation therapy [19]. Furthermore, increased serum levels of endogenous IL-6 were observed in mice treated with 5-fluorouracil, which also points to the possible role of this cytokine in the recovery of the immune and hematopoietic systems following hematopoietic stress [20]. In humans, IL-6 appeared to have a modest effect on progenitor cell mobilization, stimulating mostly platelet production [12, 21]. Human ex vivo expansion study revealed that this cytokine did participate in proliferation of HPC and the optimal growth factor combination of IL-6 with IL-3, G-CSF, GM-CSF and SCF was identified for the expansion of both total nucleated cells and early hematopoietic cells [22]. Studies in vitro also demonstrated that IL-6 was a permissive factor for monocytic colony formation by human HPC and its paracrine production by bone marrow monocytes was responsible for this process [23]. Furthermore, recent studies have shown that both proliferation and differentiation of human HPC could be induced by IL-6-IL-6sR complex, through gp 130 signalling on stem cells, in the presence of SCF [24]. A significant expansion of all hematopoietic progenitors as well as CD34+ cells was noted in the presence of IL-6, IL-6sR and SCF, whereas neither G-CSF, IL-3, GM-CSF nor EPO were able to synergize with IL-6 and IL-6sR. This, again, might have important implications in hematopoiesis following surgery when increased levels of IL-6 and IL-6sR were present in the blood circulation.

Recent studies have revealed that IL-8 possessed progenitor cell mobilization properties [25]. Injected to rhesus monkeys in the dose of 100 μg/kg of body weight, which resulted in peak plasma levels up to 5 μg/ml, it caused 10- to 100-fold increase in the numbers of circulating hematopoietic progenitor cells at 30 min and a return to the normal values at 240 min after injection. Besides, a 10-fold elevation of neutrophils was seen within 30 min of IL-8 injection. Since a significant elevation in IL-8 plasma levels was observed in patients following elective cholecystectomy, it seems that IL-8 might also be responsible for some increases in the numbers of circulating HPC after surgery.

Taken together, a release of stem and myeloid lineage CD34+, TdT+, CD38+, CD13+, CD14+ and CD33+ cells, as well as high concentrations of blood IL-6, IL-6sR and IL-8 after surgical trauma, point to an immediate stimulation of bone marrow by damaged tissue products and release of bone marrow hematopoietic precursors which presumably participate in the reconstructive process. It remains unknown what role the circulating CD34+ play after surgical trauma in otherwise hematopoietically competent subjects. Do these cells migrate to the peripheral lymphatic organs draining the damaged tissues or to the damaged tissues themselves in order to enhance the local cellular reaction?

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